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ORIGINAL ARTICLE

Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation

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Abstract

AIM: To investigate the effects of the heme oxygenase (HO)-1/carbon monoxide system on iron deposition and portal pressure in rats with hepatic fibrosis induced by bile duct ligation (BDL).

METHODS: Male Sprague-Dawley rats were divided randomly into a Sham group, BDL group, Fe group, deferoxamine (DFX) group, zinc protoporphyrin (ZnPP) group and cobalt protoporphyrin (CoPP) group. The levels of HO-1 were detected using different methods. The serum carboxyhemoglobin (COHb), iron, and portal vein pressure (PVP) were also quantified. The plasma and mRNA levels of hepcidin were measured. Hepatic fibrosis and its main pathway were assessed using Van Gieson's stain, hydroxyproline, transforming growth factor-β1 (TGF-β1), nuclear factor-E2-related factor 2 (Nrf2), matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1).

RESULTS: Serum COHb and protein and mRNA expression levels of HO-1 and Nrf2 were increased in the BDL group compared with the Sham group and were much higher in the CoPP group. The ZnPP group showed lower expression of HO-1 and Nrf2 and lower COHb. The levels of iron and PVP were enhanced in the BDL group but were lower in the ZnPP and DFX groups and were higher in the CoPP and Fe groups. Hepcidin levels were higher, whereas superoxide dismutase levels were increased and malonaldehyde levels were decreased in the ZnPP and DFX groups. The ZnPP group also showed inhibited TGF-β1 expression and regulated TIMP-1/MMP-2 expression, as well as obviously attenuated liver fibrosis.

CONCLUSION: Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and regulating PVP.

Key words: Heme oxygenase-1; Hepcidin; Iron accumulation; Oxidative stress; Portal vein pressure; Carboxyhemoglobin; Bile duct ligation

Core tip: In this study, inhibiting heme oxygenase-1 (HO-1)/carbon monoxide (CO) system by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation, the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the nuclear factor-E2-related factor 2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining portal vein pressure.

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INTRODUCTION

Iron is an essential nutrient for growth and survival, but excessive iron accumulation in cells can result in cell injury[1,2]. Iron overload is not uncommon in many patients with end-stage liver cirrhosis, and it can also occur in patients with a history of multiple blood transfusions[3,4].

Research has shown that in cultured hepatocytes, iron activates hepatic stellate cells and increases the secretion of latent transforming growth factor-β (TGF-β) due to hepatocytes being injured by iron in the pathogenesis of iron-induced liver fibrosis[5]. In mice, iron overload enhanced the development of carbon tetrachloride-induced hepatic fibrosis[6]. In clinical studies, approximately half of patients with hereditary iron accumulation (hemochromatosis) developed liver fibrosis[7]. Moreover, a significant reduction of fibrosis in the liver was demonstrated in a number of thalassemia patients treated with deferasirox[8].

Clinically, repeated large-volume blood transfusions are sometimes necessary for cirrhotic patients with massive upper gastrointestinal bleeding; in most cases, patients are transfused with packed red blood cells (RBCs), which results in iron overload as the human body cannot excrete iron. Each unit of RBCs contains approximately 250 mg of iron, and after 10-15 RBC transfusions, iron typically accumulates in the liver, heart, skin, and endocrine organs[9]. However, how iron overload affects the pathogenesis and treatment of patients with hepatic fibrosis is not yet well understood.

Heme oxygenase-1 (HO-1) is the primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme into free iron, carbon monoxide (CO), and biliverdin[10,11].

Previous reports have recently shown HO-1 to be protective in liver cells in various liver diseases such as acute liver injury, alcoholic liver disease, liver fibrosis and ischemia/reperfusion injury through multiple pathways[12-15]. Other reports have indicated that this protection might be restricted to a narrow threshold of HO-1 over-expression[13,16]. Our previous studies showed that over-expression of HO-1 could be harmful to the liver functioning of rats with cirrhosis induced by bile duct ligation (BDL)[17,18], which was also reported by Froh *et al*[19], but whether this effect was related to iron accumulation and CO release was not clear.

In normal Sprague-Dawley (SD) rats, increased HO activity as a pro-oxidant mechanism resulted in iron accumulation in the liver; in contrast, decreased HO activity reduced intracellular iron levels and oxidative stress[20]. In this study, we investigated the effect of HO-1 on iron accumulation and CO release by inhibiting or inducing HO-1 expression with zinc protoporphyrin (ZnPP) or cobalt protoporphyrin (CoPP) in fibrotic rat models induced by BDL, and we further studied whether regulating HO-1 expression could improve liver fibrosis by reducing hepatic iron accumulation and portal vein pressure (PVP).

MATERIALS AND METHODS

Animal care

The experimental protocols were approved by the Animal Care and Use Committee of Dalian Medical University (Liaoning Province, China), in accordance with the guidelines established by the Canadian Council on Animal Care.

BDL and treatment in rat

Fifty-three healthy male SD rats, weighing 200-220 g, were obtained from the Laboratory Animal Center of Dalian Medical University and were randomly divided into six groups: a Sham group (n = 6), BDL group (n = 10), CoPP treatment group (n = 12), ZnPP treatment group (n = 8), Fe treatment group (n = 9) and DFX treatment group (n = 8). The rats were housed in a specific pathogen-free (SPF) center, at room temperature of 24-26 ℃ and relative humidity of 60%-65%. Water was provided ad libitum.

The rats were well fed and housed for 3 d before any experimental protocols. Biliary cirrhosis was induced by BDL[21,22]. Five groups underwent BDL together with Sham-operated animals as a healthy control. The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with 4-0 silk sutures, and the antibiotic gentamicin (0.3 mL) was injected intramuscularly. Rats in the Sham group underwent laparotomy with the bile duct isolated but not ligated. After surgery, the Sham and BDL groups received an intraperitoneal injection of saline. Other groups received an intraperitoneal injection consisting of CoPP, ZnPP, iron-dextran (ID) and deferoxamine (DFX) (5, 5, 50, 100 mg/kg body weight) three times per week, respectively. After the establishment of the rat models, the number of rats was reduced to 6 in each group because of deaths during the study process.

ZnPP and CoPP (Sigma, St Louis, MO, United States) were dissolved in 0.2 mol/L of NaOH, adjusted to a pH of 7.4, were diluted in 0.85% NaCl, with a final concentration of 1 mg/mL as previously described, and were used for inhibiting and inducing HO-1 expression, respectively[23]. DFX mesylate salt and ID (Sigma, St Louis, MO, United States) were diluted in 0.85% NaCl with final concentrations of 40 and 20 mg/mL, respectively. Histostain™ - Plus Kits (SP9001) (Zhongshan Goldenbridge

Biological Technology, Beijing, China); hydroxyproline (HYP), malonaldehyde (MDA) and superoxide dismutase (SOD) (Key GEN Biotech Nanjing, China); a hepcidin enzyme-linked immunosorbent assay (ELISA) Kit (EIAab Science, Wuhan, China); and a TaKaRa RNA polymerase chain reaction (PCR) kit (avian myeloblastosis virus), version 3.0 (TaKaRa Biotechnology, Dalian, China), were used in this study.

Sample collection and examination

Four weeks after surgery, a catheter connected to a pressure transducer (BL-420F biological experimental system, Chengdu Technology and Market Co. Ltd., China), was placed in the portal vein to measure PVP. Subsequently, 1 mL of arterial blood was withdrawn to measure carboxyhemoglobin (COHb), using a Rapid Lab 1245 Blood Gas Analyzer (Siemens, New York, NY, United States). Then, blood samples were collected from the abdominal aorta to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and serum iron, using a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co, Tokyo, Japan). The levels of liver SOD and MDA were determined with a UV-2100 spectrophotometer (Chemito Instruments Pvt. Ltd.).

Liver iron content measurement

Liver iron content was determined by atomic absorption spectrometry with acetylene-air flame atomization. The analysis was performed with a Varian atomic absorption spectrometer (Mulgrave) with deuterium background correction. Measurements were obtained with a 248.3 nm analytical line in the spectral interval of 0.2 nm. Iron concentration was determined by the standard addition method. Sample digestion was accomplished with the MDS 2000 microwave sample preparation system (CEM) in Teflon cartridges, using a mixture of nitric acid (5 mL) and hydrogen peroxide (2 mL) (both from Merck, ultrapure grade) for 20 min at a pressure of 120 psi. The resulting product was analyzed directly in the Teflon cartridges.

Histology and immunohistochemistry

Part of liver lobe was excised, and the tissue was fixed in 10% neutral formalin solution and embedded in paraffin. Hematoxylin and eosin staining and Van Gieson's (VG) staining were performed according to standard procedures. The severity and degree of lesions were graded according to methods previously described[24,25]. Briefly, tissue sections (4 μm thick) were treated with HCl (5%) to liberate ferric ions. The samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. The slides were counterstained with Neutral red. For immunohistochemical examination, deparaffinized sections were incubated with HO-1 antibodies (1:1000 dilution) and appropriate biotinylated secondary antibodies, followed by the avidin-biotin-peroxidase complex. The immunoreactive signal was developed by color deposition, using diaminobenzidine as a substrate. Yellow material in the cytoplasm was considered to indicate positive cells. Cell staining was assigned 4 scores, as previously described[26]. The final score was defined as staining intensity × percentage of positive cells. The mean score of five fields was used to compare the six groups.

Hepatic HYP content

Liver tissue (100 mg) was prepared for HYP determination, according to a modification of the method previously described[27]. The HYP content of the liver, as an indirect measurement of tissue collagen content, was expressed in microgram per gram of wet weight (μg/g).

Measurement of plasma hepcidin

Plasma hepcidin was measured by ELISA and was determined using 96-well microtiter plates coated with the recombinant peptide and a polyclonal antibody (Santa Cruz Biotechnology, INC, 1:3000 dilution). Assay procedures were performed according to the manufacturer's instructions, and absorbance of each well was determined at a 450 nm wavelength. The process was performed as described previously[28].

Western blot analysis

The resected hepatic tissues were extracted with lysis buffer (1% Triton X-100; 50 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; and 1% protease inhibitor cocktail). The protocols for western blot analyses have been described previously[29]. Western blot analyses were performed with liver homogenates (30 μg protein) using anti-nuclear factor-E2-related factor 2 (Nrf2) antibody (Boster Biological Technology, Wuhan, China, 1: 100 dilution), anti-TGF-β1 antibody (Boster Biological Technology, Wuhan, China, 1:100 dilution), anti-HO-1 antibody (Abcam, Cambridge, MA, United States, 1:2000 dilution), anti-β-actin antibody (Zhongshan Goldenbridge Biological Technology, Beijing, China, 1:500 dilution), and secondary antibody anti-rabbit and anti-mouse immunoglobulin G (Biosynthesis Biotechnology, Beijing, China, 1:500 dilution). The intensity of each signal was corrected using the values obtained from the immunodetection of β-actin, and the relative protein intensity was expressed as multiples of the content in the normal group.

RNA isolation and gene expression analysis

Total RNA was extracted from the livers following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm (A_{260} = 1 for 40 μg/mL RNA), and the purity of RNA was assessed by determining the ratio of the optical density obtained at 260 and 280 nm (pure RNA: A_{260}/A_{280} = 2.0) using a UV-1206 spectrophotometer (Shimadzu, Japan). An aliquot of each mixture was used for reverse-transcription (RT)-PCR amplification, using reagents purchased from Takara Bio Inc. (Dalian, China). PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were

Table 1 Primers used for the reverse transcription-polymerase chain reaction and polymerase chain reaction analysis

Gene	Gene ID	Forward/reverse	Sequences 5'-3'	Product size (bp)
HO-1	NM012580	Forward	ATATCTATACGGCCCTGGAA	350
		Reverse	GATGCTCGGGAAGGTGAA	
Nrf2	AF304364	Forward	GACGGCAACACTGATTCCA	345
		Reverse	CATCCGCCACTCATTCCT	
TGF-β1	NM021578	Forward	CCGCAACAACGCAATCTA	437
		Reverse	TGAGGAGCAGGAAGGGTC	
Hepcidin	NM053469	Forward	GCTGCCTGTCTCCTGCTT	159
		Forward	GGTGTCTCGCTTCCTTCG	
TIMP-1	NM053819	Forward	CTCTGGCCTCTGGCATCCT	300
		Reverse	ACTCCTCGCTGCGGTTCT	
MMP-2	NM031054	Forward	CTGGGCAACAAGTATGAGA	430
		Reverse	CTGTCCGCCAAATAAACC	
β-actin	NM031144	Forward	GAGGGAAATCGTGCGTGAC	445
		Reverse	CTGGAAGGTGGACAGTGAG	

HO-1: Heme oxygenase-1; Nrf2: Nuclear factor-E2-related factor 2; TGF-β1: transforming growth factor-β1; TIMP-1: Tissue inhibitor of metalloproteinase-1; MMP-2: Matrix metalloproteinase-2.

photographed, and the density of each product band was quantified. The results are expressed as the ratios of the band density for target mRNA to that of β-actin mRNA. The primers utilized for PCR and RT-PCR are listed in Table 1.

Statistical analysis

All of the data are presented as the mean ± SD. Statistical testing was performed with SPSS software, version 16.0. The groups were compared using one-way analysis of variance with Dunnett's multiple comparison test (where applicable). Correlative comparison of two non-hierarchical variances with normal distribution was evaluated by Pearson's test, whereas Spearman's test was used for non-normally distributed data. $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of biochemical indicators in liver fibrosis induced by BDL

Four weeks postoperatively, common bile duct dilatation was observed in the BDL group, and ascites and jaundice also developed in the BDL group, suggesting that the BDL model was successfully established in our experiments.

The serum levels of AST, ALT and TBIL in the BDL group were much higher than those in the Sham group ($P < 0.01$). These levels were much lower in the ZnPP group and DFX group, but the levels in the CoPP group and Fe group were significantly higher CoPP than those in the BDL group ($P < 0.01$) (Figure 1G and H). The serum levels of AST were decreased in the DFX group compared with those in the ZnPP group ($P < 0.05$) (Figure 1G). These data indicated that inhibiting HO-1 expression and further reducing iron accumulation could improve liver function; in contrast, inducing HO-1 expression aggravated liver injury.

Inhibiting HO-1 expression reduced liver fibrosis and PVP induced by BDL

Liver damage was analyzed by hematoxylin and eosin staining. The livers in the Sham group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation were found in the interlobular and central venous regions in the livers in the BDL and CoPP groups (Figure 1B and C). Compared with the BDL group, fibrous hyperplasia was significantly reduced around the central veins in the ZnPP group (Figure 1D). The histopathological scores for fibrosis in the livers of BDL rats were improved in the ZnPP group (Figure 1I). Collagen type I was observed with VG staining (Figure 2A-F). In the BDL group, collagen type I in the portal area and bile duct wall was much thicker than in the Sham group ($P < 0.01$) (Figure 2A and B). Compared with the BDL group, there was a decrease in type I collagen in the ZnPP group. The extent of fibrosis was much higher in the CoPP group than in the BDL group ($P < 0.01$) (Figure 2G). The change in HYP content in liver tissue was in accordance with type I collagen. It was observed that HYP was significantly decreased in the ZnPP group compared with the BDL group (Figure 2H). These data showed that inhibiting HO-1 expression reduced collagen deposition in liver fibrosis.

The COHb levels in arterial blood were significantly higher in the BDL group compared with the Sham group Sham ($P < 0.01$), and they were much lower following ZnPP and DFX treatment, while they were higher in the CoPP- and Fe-treated groups than in the BDL group ($P < 0.01$) (Figure 3A). PVP was significantly higher in the BDL group compared with the Sham group ($P < 0.01$). Compared with the BDL group, PVP decreased in the ZnPP and DFX groups ($P < 0.01$) and was enhanced in CoPP and Fe rats ($P < 0.01$). Moreover, PVP decreased following ZnPP treatment relative to DFX treatment ($P < 0.05$) (Figure 3B).

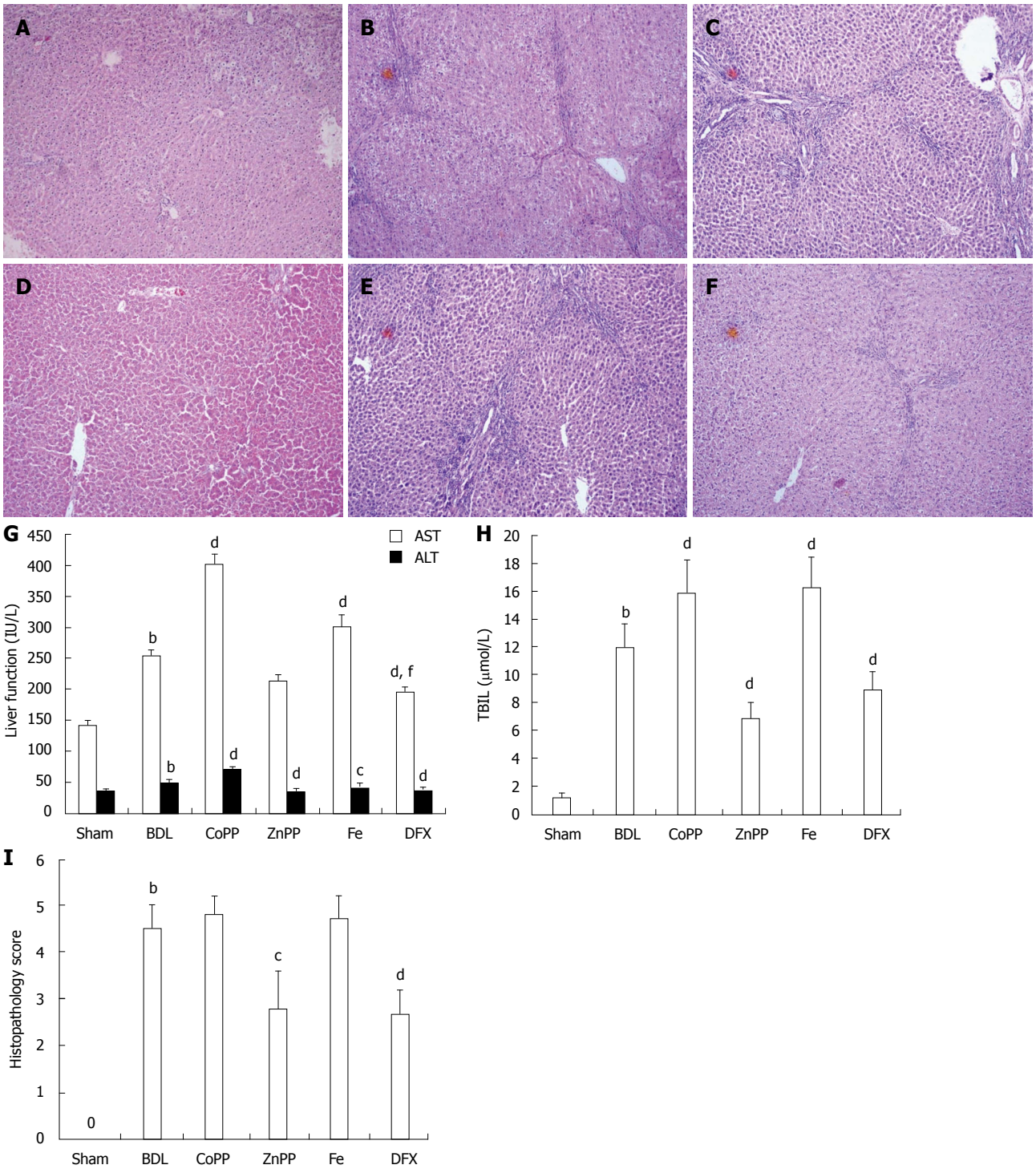


Figure 1 Pathological features of rat liver tissue detected by hematoxylin and eosin staining and serum index. A: Normal lobular architecture in the Sham group; B, C, E: Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D and F: Less fibrous hyperplasia and fibrosis in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; G, H: Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (TBIL); I: Histopathological scores for fibrosis (magnification × 100). Values are expressed as mean ± SE (n = 6). $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* BDL group; $^{f}P < 0.01$ *vs* ZnPP group.

Levels of TGF-β1 were significantly enhanced in the BDL group compared with the Sham group ($P < 0.01$). These levels were lower in the ZnPP group and higher in the CoPP group compared with the BDL group (Figure 4A and B). The mRNA expression levels of MMP-2 and TIMP-1 were much higher in the BDL group than in the Sham group ($P < 0.01$). These levels were significantly lower in the ZnPP group and higher in the CoPP group than in the BDL group ($P < 0.01$) (Figure 4C). These results showed that down-regulated HO-1 expression reduced extracellular matrix (ECM) deposition and fibrosis.

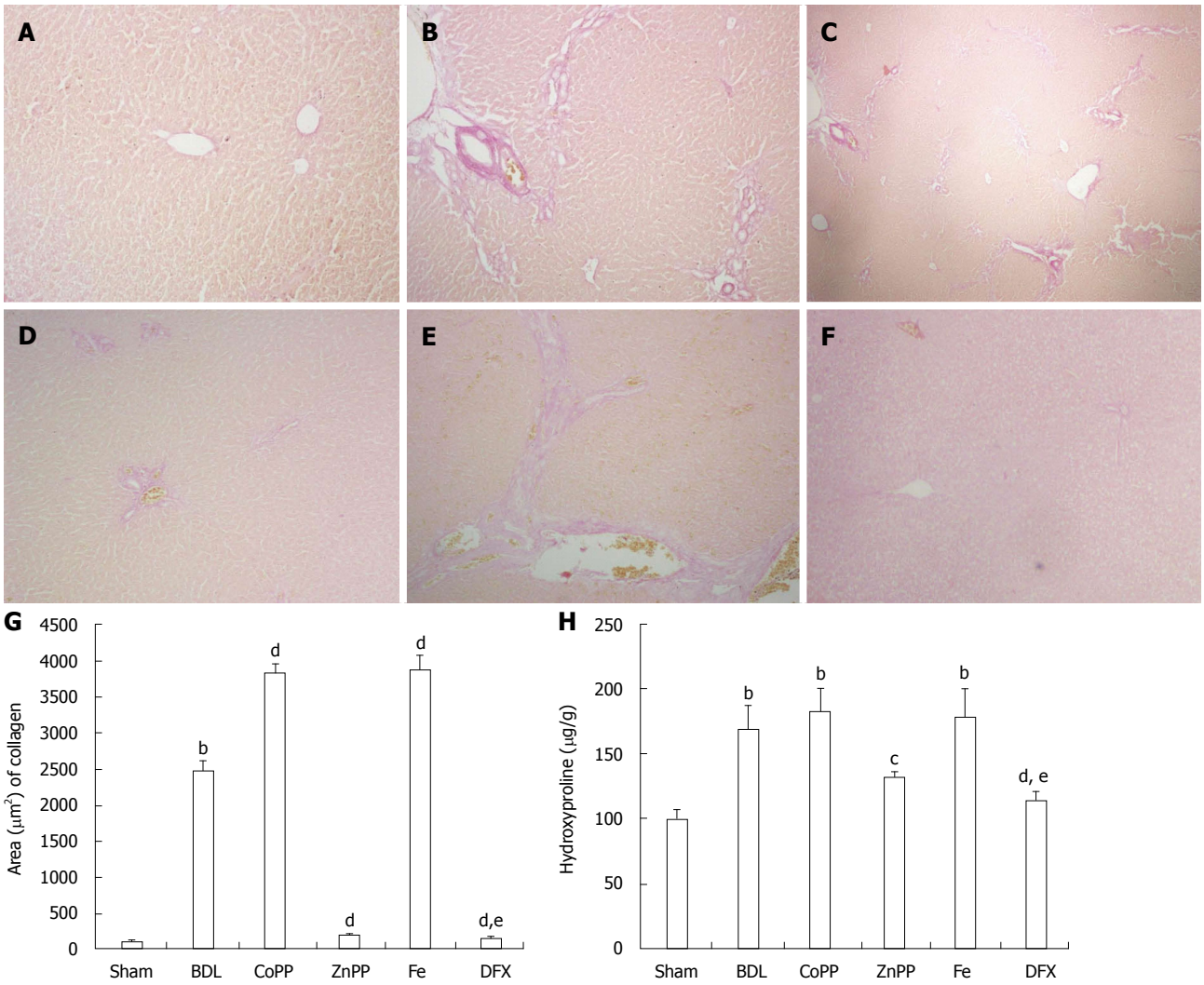


Figure 2 Van Gieson's staining of collagen Ⅰ for liver sections and liver hydroxyproline content. A-F: Collagen type Ⅰ was deposited in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group (B, C and E) and was rarely found in the Sham group, zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group (A, D and F); G: The area of collagen type Ⅰ; H: Hydroxyproline content of liver tissue (magnification, × 100). Values are expressed as mean ± SE (n = 6). $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* BDL group; $^{e}P < 0.05$ *vs* ZnPP group.

HO-1 mediated iron accumulation and oxidative stress in liver

The mRNA and protein expression levels of HO-1 were significantly higher in the BDL group than in the Sham group ($P < 0.01$). These levels were obviously lower in the ZnPP group and higher in the CoPP group than in the BDL group ($P < 0.01$) (Figure 4A and B). Hepatic immunostaining showed that HO-1 was mainly expressed in the liver cells and partly in the mesenchymal cells and Kupffer cells. Localization of HO-1 occurred mainly around the centrilobular veins (Figure 5A-F). The values of HO-1 expression were consistent with the above data (Figure 5G).

The serum levels of iron in the BDL group were significantly higher than in the Sham group ($P < 0.01$). These levels were greatly lower in the ZnPP group than in the BDL group ($P < 0.01$) (Figure 6G). The change in liver iron content was in accordance with serum iron levels (Figure 6H).

The mRNA and plasma levels of hepcidin were significantly lower in the BDL group than in the Sham group ($P < 0.01$). These levels were higher in the ZnPP group and lower in the CoPP group compared with the BDL group, and they were higher in the DFX group than in the ZnPP group (Figure 4C and 6I).

We used Prussian blue staining to localize iron accumulation in liver tissue and found that iron obviously accumulated in the BDL and CoPP groups. Iron was strongly stained mainly in Kupffer cells in these groups (Figure 6B and C). However, iron staining was rarely found in the Sham and ZnPP groups (Figure 6A and D). These results indicate that inhibiting HO-1 expression could reduce iron production, resulting in decreased iron accumulation in the liver. In contrast, enhanced HO-1 expression led to increased hepatic accumulation of iron.

Levels of SOD were obviously lower in the BDL group than in the Sham group ($P < 0.01$), and they were significantly higher in the ZnPP group and lower in the CoPP group than in the BDL group ($P < 0.01$) (Figure 3C). The MDA change tendency was opposite that of

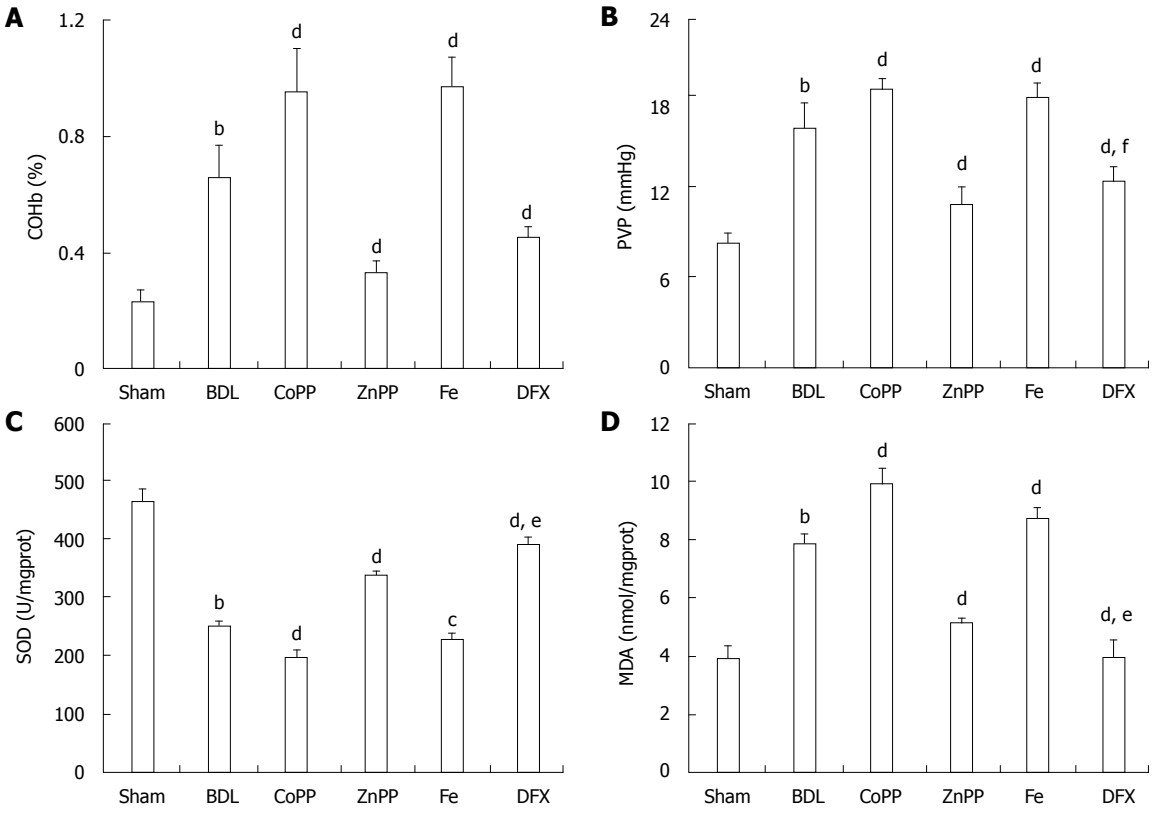
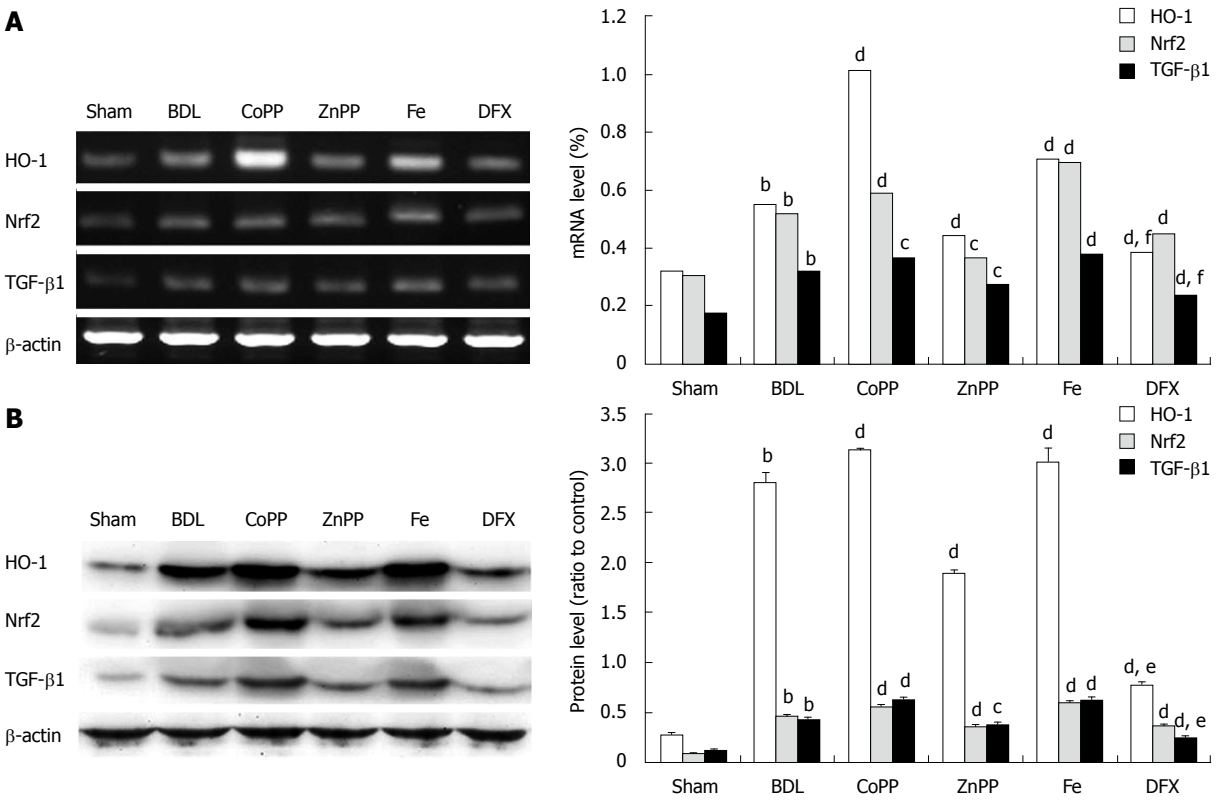


Figure 3 The levels of carboxyhemoglobin, portal vein pressure malonaldehyde and superoxide dismutase. A, B: The levels of carboxyhemoglobin (COHb) were accordance with heme oxygenase-1 expression, and portal vein pressure (PVP) levels were measured; C, D: The levels of superoxide dismutase (SOD) and malonaldehyde (MDA) was detected. Values are expressed as mean ± SE (n = 6). $^{b}P < 0.01$ *vs* Sham group; $^{d}P < 0.01$ *vs* BDL group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* zinc protoporphyrin (ZnPP) group. BDL: Bile duct ligation; CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.



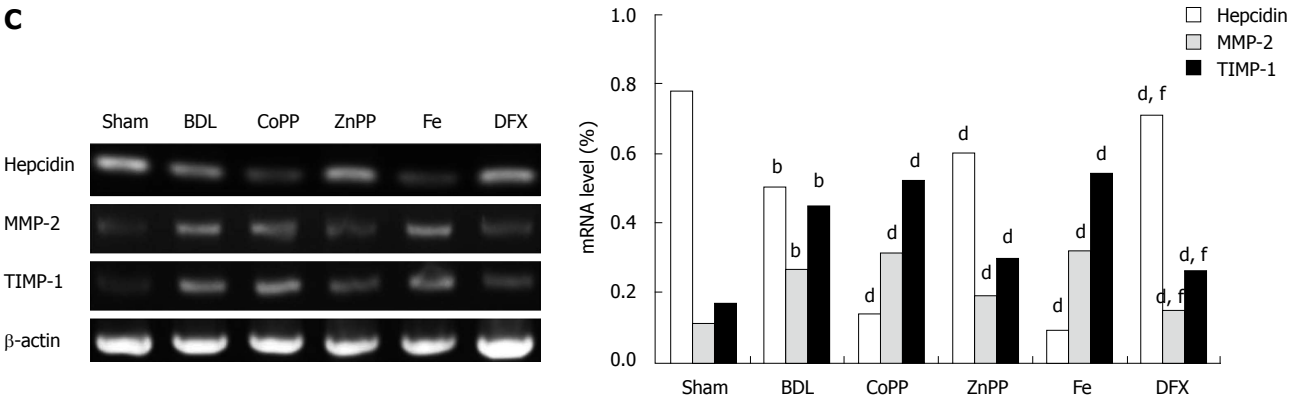


Figure 4 The heme oxygenase-1, transforming growth factor-β1, nuclear factor-E2-related factor 2, hepcidin, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression were detected by Western blot and reverse transcription-polymerase chain reaction. A, B: The mRNA and protein levels of heme oxygenase-1 (HO-1), transforming growth factor-β1 (TGF-β1) and nuclear factor-E2-related factor 2 (Nrf2); C: The mRNA levels of hepcidin, matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1). Values are expressed as mean ± SE. $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* bile duct ligation (BDL) group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* zinc protoporphyrin (ZnPP) group. CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.

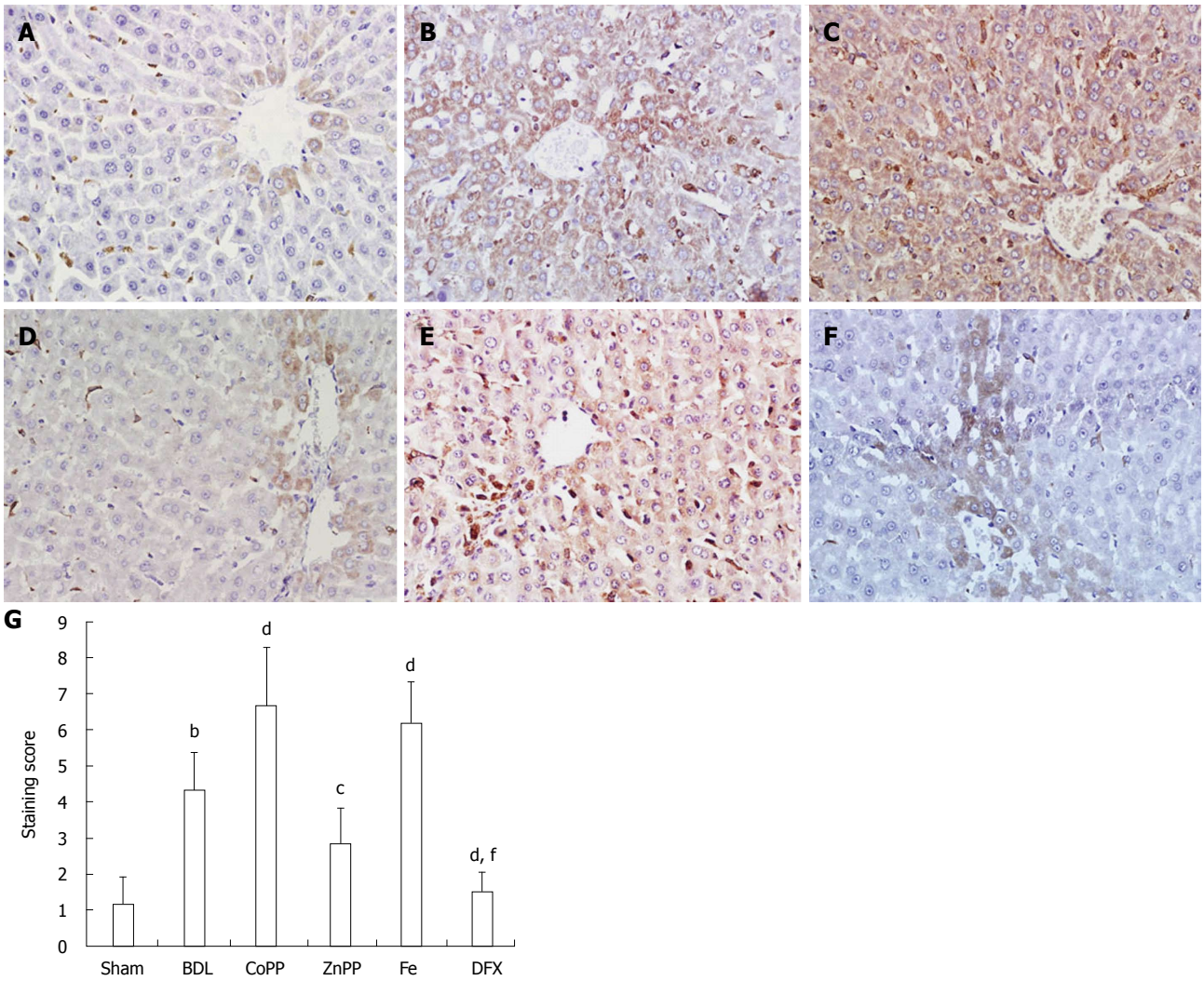


Figure 5 Liver sections were stained with heme oxygenase-1 antibody. A: Heme oxygenase-1 (HO-1) expression was less around the central veins in the Sham group; B, C, E: Much more HO-1 expression was found around the central veins in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D, F: Less staining was observed in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; H: Immunohistochemical staining scores (magnification ×400). Values are expressed as mean ± SE ($n = 6$). $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* BDL group; $^{f}P < 0.01$ *vs* ZnPP group.

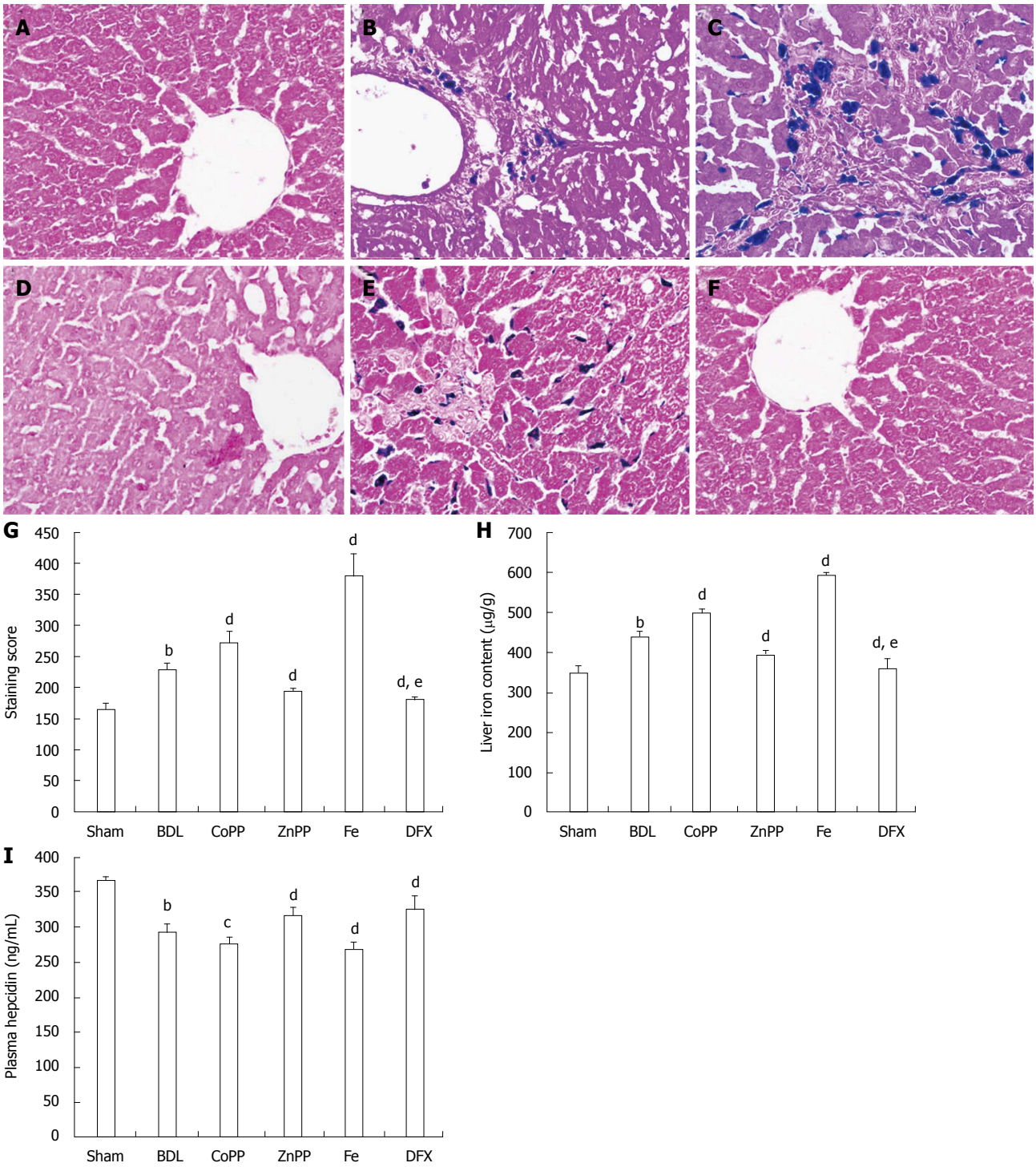


Figure 6 Perl's Prussian blue staining, levels of hepcidin, serum and liver iron. A: No iron accumulated in the Sham group; B: A small amount of iron mainly accumulated on Kupffer cells in the bile duct ligation (BDL) group; C: Much more iron accumulation was found in interlobular and macrophagocytes in the cobalt protoporphyrin (CoPP) group; D, F: Almost no iron accumulation was detected in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; E: Massive iron accumulation was observed in the Fe group; G, H: There were no differences in the hepatic and serum iron content of these six groups; I: Plasma hepcidin also was measured by enzyme-linked immuno sorbent assay (magnification × 400). Values are expressed as mean ± SE (n = 6). $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* BDL group; $^{e}P < 0.05$ *vs* ZnPP group.

SOD (Figure 3D). In the BDL model, inhibiting HO-1 expression reduced oxidative stress.

Iron induced oxidative stress and Nrf2 expression

Both the serum iron and liver iron content were higher in the Fe group but lower in the DFX group compared with the BDL group (Figure 6G and H). Prussian blue staining showed more iron accumulation in the Fe group and less in the DFX group (Figure 6E and F). The ZnPP group showed low iron levels (Figure 6D and F-H). The levels of plasma hepcidin were obviously lower in the Fe group but higher in the DFX group compared with the

BDL group (Figure 6I). The expression of HO-1 was significantly higher in the Fe group but was lower in the DFX group compared with the BDL group ($P < 0.01$). It was also lower in the DFX group than in the ZnPP group (Figure 4A and B).

The mRNA and protein expression levels of Nrf2 were enhanced in the BDL group compared with the Sham group. Additionally, these levels were significantly higher in the CoPP and Fe groups than in the BDL group, and they were lower in the ZnPP and DFX groups ($P < 0.01$) (Figure 4A and B). We found the levels of SOD in the Fe group to be slightly lower than in the BDL group ($P < 0.05$); however, they were much higher than in the DFX group ($P < 0.01$) (Figure 3C). The levels of MDA were significantly increased in the Fe group but were reduced in the DFX group compared with the BDL group ($P < 0.01$) (Figure 3D). The levels of SOD were much higher, and MDA was lower in the DFX group compared with the ZnPP group ($P < 0.01$) (Figure 6C and D). These data indicate that iron accumulation in the liver increased the oxidative stress reaction and caused further damage to the liver.

Hematoxylin and eosin staining showed more fibrous hyperplasia in the Fe group and less in the DFX group compared with the BDL group (Figure 1E and F). The content of HYP was significantly higher in the Fe group than in the Sham group ($P < 0.01$), and it was lower in the DFX group than in the BDL group ($P < 0.01$) (Figure 2H). Compared with the BDL group, collagen I was increased in the Fe group and decreased in the DFX group (Figure 2E-G). The mRNA and protein expression levels of α-smooth muscle actin and TGF-β1 were significantly enhanced in the Fe group and decreased in the DFX group compared with the BDL group (Figure 4A and B). The levels of MMP-2 and TIMP-1 mRNA were much higher in the Fe group and were lower in the DFX group compared with the BDL group ($P < 0.01$). Compared with the ZnPP group, TGF-β1 expression and ECM were lower in the DFX group (Figure 4C).

Correlation between oxidative stress and liver fibrosis

Correlation analysis revealed that both SOD and MDA were significantly correlated with HYP levels ($R = -0.912$, 0.887, respectively, $P < 0.01$). These data also showed that oxidative stress could result in ECM deposition in the liver and could further aggravate liver fibrosis.

DISCUSSION

Many chronic liver diseases progress to hepatic fibrosis[30]. Iron overload in the liver increased the risk of developing fibrosis, as well as subsequent morbidity and mortality[31]. HO-1 catalyzes heme into iron, and it plays an important role in iron homeostasis. A previous study showed that HO-1 was associated with hepatocellular damage and had multiple mechanisms to influence liver fibrosis progression. In this study, we aimed to investigate how iron and CO, the product affected by HO-1 activity, affected hepatic fibrosis and PVP. We found that lower HO-1 expression could reduce iron accumulation and PVP and improve fibrosis.

In several chronic liver diseases, HO-1 plays a protective effect in the liver against oxidative stress-dependent damage[32-34]. However, its protective effects in inflammation and fibrosis have been disputed. Some studies have shown that HO-1 over-expression increases liver injury in rats under conditions of experimental chronic cholestasis[19]. Low HO-1 induction was shown to be cytoprotective, and high levels of HO-1 could result in the accumulation of free divalent iron, thus increasing oxidative injury in fibroblast cell cultures[35]. We found that lower HO-1 expression could benefit end-stage liver cirrhosis by reducing iron accumulation, which is accordance with the findings of the above studies. Surprisingly, induction of HO-1 interfered with chronic inflammation and prevented progression of liver fibrosis in Mdr2-knockout mice, and it further might delay progression to hepatocellular carcinoma[33]. Our previous study indicated that induction of HO-1 could ameliorate immune liver fibrosis[36]. The reason why the above studies are different from this study could be that HO-1 plays a diverse role in different stages during the progression of liver fibrosis. In early stages of liver fibrosis, inducing HO-1 could have a protective effect, but it could increase liver injury in end stages *via* liver hypertension. Moreover, the different animal models for inducing fibrosis could constitute another explanation of these results.

The majority of endogenous CO is catalyzed by inducible expression of HO-1. CO can modulate blood flow and maintain the integrity of the vessel wall[37]. COHb levels can be used to estimate HO activity in experimental animals. Interestingly, we observed that up-regulated COHb resulted from increased HO-1, which aggravated PVP in BDL rats. Moreover, lower levels of COHb can decrease the PVP found in the ZnPP and DFX treatment groups. HO/CO plays a role in the pathophysiology of portal hypertension, and CO can regulate the intrahepatic vascular resistance of cirrhotic rats[38]. Tarquini *et al*[39] indicated that the HO/CO system is activated in patients with liver cirrhosis, and CO contributes to the hyperdynamic circulatory syndrome. CO might improve intrahepatic microcirculation in early stage hepatic fibrosis, and excessive CO could be harmful, leading to an unbalanced nitric oxide/CO system in end-stage hepatic fibrosis. It therefore seems best to reduce PVP by decreasing CO.

Normally, HO-1 is only slightly expressed in hepatocytes and Kupffer cells. In hepatic cirrhosis, the expression of HO-1 is increased. Khan *et al*[20] reported that an increase in HO-1 expression is associated with iron accumulation. The study of Kartikasari *et al*[40] showed that iron is derived from intracellular heme degradation, and HO-1 activity contributes to increased levels of intracellular labile iron. Other research has shown that non-heme iron increases are associated with the induction of HO-1 in neurons, microglia and capillary endothelial cells, whereas HO-2 levels remain unchanged, implying that the non-heme iron increases might be the result

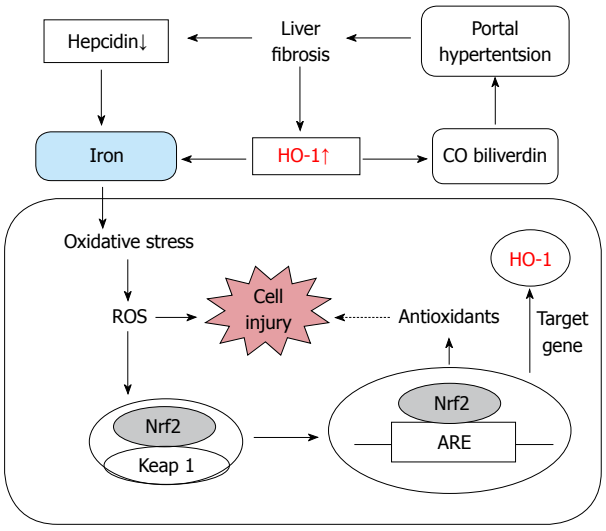


Figure 7 Iron is involved in the heme oxygenase-1 cycle *via* the nuclear factor-E2-related factor 2/Keap1 pathway, and heme oxygenase-1 regulates portal pressure in liver cirrhosis. Low hepcidin and heme oxygenase-1 (HO-1) over-expression could mediate iron accumulation, which accelerates oxidative stress, leading to cell injury, and it also increases HO-1 expression through the nuclear factor-E2-related factor 2 (Nrf2)/Keap1 pathway. Nrf2 protects cells against oxidative stress, but this effect is limited, and Nrf2 contributes to induction of HO-1 expression, which can produce iron. HO-1 can increase carbon monoxide (CO) production, and an unbalanced CO/nitric oxide system could play a role in portal pressure. ROS: Reactive oxygen species. ARE: Antioxidant response element.

of HO-1-mediated heme degradation[41]. These results showed that HO-1 played a central role in maintaining iron homeostasis *in vivo*. In this study, we found that serum iron and liver iron contents all increased in the CoPP group, and inhibiting HO-1 activity with ZnPP reduced iron accumulation in the liver and further attenuated liver fibrosis in liver fibrosis induced by BDL.

Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum. It was also noted that hepcidin was abnormally low in alcoholic patients with associated iron overload[42]. Iron was accumulated in the liver and pancreas of hepcidin-deficient mice[43]. It also was found that serum pro-hepcidin concentrations were lowered in liver cirrhosis, which could be the result of impaired liver functioning[44]. Hepcidin is down-regulated during progressive cholestasis in biliary atresia[45-47]. Furthermore, Huang *et al*[48] showed that iron loading down-regulates hepcidin by inhibiting both inflammatory and iron-sensing pathways and inhibiting transducers and activators of transcription 3 and SMAD4 signaling *in vivo*. These findings are consistent with the results of our experiment. Under physiological conditions, hepcidin expression is stimulated by iron overload and inflammation and is suppressed by anemia and tissue hypoxia[49,50]. However, levels of hepcidin were decreased in the iron accumulation group and were increased in the ZnPP and DFX groups in our study. The reason for this finding might have been the various signals affecting hepcidin production. Up-regulation of hepcidin by inhibiting HO-1 expression could be beneficial for cholestasis in cirrhosis.

It is now commonly accepted that HO-1 plays an important role in the control of inflammation and oxidative stress[51]. HO-1 protected primary human hepatocytes from ethanol-derived oxidative stress *via* the MAPK/Nrf2 pathway[52]. Surprisingly, however, in this study, we found that inducing HO-1 expression increased MDA and decreased SOD. Further, these results indicate that HO-1 could not reduce the oxidative stress reaction. Other studies have shown the pro-oxidant nature of the released cellular low-molecular-mass iron and the antioxidant effect of the released bilirubin[53]. In this study, we demonstrated that the pro-oxidant activities of iron accumulation were much stronger than the antioxidant effects of bilirubin.

Iron primarily accumulates in the reticuloendothelial cells. Previously, it was shown that increased deposition of iron in the liver often triggered oxidative stress and inflammation and induced liver cell damage[54]. It can participate in Fenton and Haber-Weiss chemistry, and excessive redox-active iron might lead to oxidative stress, with damage to membranes, proteins and DNA[55]. It was also shown that increased deposition of iron in the liver induced liver cell damage and cirrhosis by triggering oxidative stress and inflammation[56]. In fact, signs of iron-catalyzed lipid peroxidation and oxidative stress have been found by many investigators during chronic iron overload in rodents[57,58]. In this study, iron intoxication dramatically enhanced MDA adducts, decreased antioxidant enzyme SOD and aggravated liver injury in the BDL, CoPP and Fe groups. Our results revealed that iron accumulation exacerbated the oxidative stress reaction, leading to the aggravation of liver cirrhosis.

Previous reports have shown that elevated hepatic iron can activate Nrf2 in 3,5,5-trimethyl-hexanoyl-ferrocene-treated mouse models[59]. In our study, Nrf2 was up-regulated in the BDL, CoPP and Fe groups, in which iron accumulation in the liver was found. Nuclear translocation of activated Nrf2 is an important upstream contributor to the induction of HO-1 expression[60]. Up-regulation of Nrf2 increased HO-1 gene transcription in the CoPP and Fe groups. The pathway of iron-dependent HO-1 induction involves Nrf2/Keap. Nrf2 also plays a key role in the protection of cells against oxidative stress[61]. However, in our study, Nrf2's protective effect was limited, and HO-1, which is its target gene, increased iron production, resulted in oxidative stress.

Previous studies have demonstrated that oxidative stress significantly contributes to hepatic fibrogenesis from various liver injuries[62]. Reactive oxygen species (ROS) can stimulate the production of type I collagen and could act as intracellular signaling mediators of TGF-β[63,64]. Our study showed that preventing oxidative stress by inhibiting HO-1 expression could attenuate liver fibrosis through regulation of the TGF-β1 pathway and reducing collagen deposition.

In conclusion, HO-1 played a pivotal role in iron accumulation and portal pressure in the livers in our study

(Figure 7). In the clinic, many end-stage cirrhosis patients with upper gastrointestinal bleeding treated with multiple massive transfusions run the risk of iron overload and further liver injury. Today, iron chelation therapy is often utilized to remove excess stored iron in some diseases[65,66]. In our study, iron accumulation induced hepatic fibrogenesis, indicating that cirrhotic patients with massive stored RBC transfusions would benefit from iron removal therapy. Our research provided a new way to reduce liver iron and portal pressure by inhibiting HO-1 expression. However, we must find a proper model for the simulation of upper gastrointestinal bleeding and transfusion. In clinical experiments, we will attempt to include cirrhotic patients with bleeding complications, who would eventually receive transfusions, to investigate the effects of iron transfusions on liver cirrhosis.

Removing iron and reducing portal pressure by inhibition of HO-1 improves liver fibrosis in bile duct-ligated rats. In addition, iron is also closely related to another complication of cirrhosis: hepatocellular carcinoma[67]. Regulation of iron homeostasis, by interfering with HO-1, could effectively treat hepatic cirrhosis and also prevent hepatocellular carcinoma.

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COMMENTS

Background

Iron overload in the liver is a very common phenomenon in many chronic liver diseases. Heme oxygenase-1 (HO-1) and its by-products, iron and carbon monoxide (CO), play crucial roles in hepatic fibrosis. The underlying molecular mechanisms of HO-1, regarding iron deposition and portal vein pressure (PVP) in hepatic fibrosis, remain unknown.

Research frontiers

HO-1 and degradation products are important to cytoprotection in many types of liver injury, but protection can be restricted to a narrow threshold. Iron overload often triggers oxidative stress and inflammation and induces liver cell damage, and the CO/nitric oxide system could be harmful to portal pressure. Iron can activate nuclear factor-E2-related factor 2 (Nrf2) and increase HO-1 expression. Inhibiting HO-1 activity is necessary for reducing iron and PVP.

Innovations and breakthroughs

In this study, by inhibiting HO-1 expression by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation (BDL), the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining PVP.

Applications

Removing iron and reducing CO by inhibiting HO-1 activity provides a new strategy for treating liver fibrosis, and further, it could help prevent liver carcinoma.

Terminology

HO-1 is a primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme to free iron, CO, and biliverdin. Nrf2 is an important upstream contributor to the induction of HO-1 expression, and it has a protective effect on cells against oxidative stress. Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum.

Peer review

This study analyzed the role of HO-1 inhibition in rat liver fibrosis using an experimental model of BDL. The results are interesting, and they suggest that regulation of iron homeostasis and CO production could effectively treat liver cirrhosis and PVP and even prevent hepatocellular carcinoma.

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